Molecular Modeling of the Interaction of Cellulose with Cellulases and Catalysts
Subcontract No. XDH-0-30009-03
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Technical Status Report
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During this period, work was performed on tasks 1,2, 4 and 5, and the deliverable for task 1 is provided in draft form.

DELIVERABLE (TASK 1): Deliver a standard protocol of the BCA method for determination of cellulase activity on insoluble high-molecular weight substrates.

Pertaining to **Task 1** of the UC Davis Statement of Work, the BCA reducing end-group analysis has been adapted for measuring activity of cellulases on insoluble high-molecular weight cellulose. A protocol of the standard procedure is attached to this report.

The method has been developed using bacterial cellulose from *Acetobacter xylinum* and endoglucanase I (EGI) from *T. reesei*. Bacterial cellulose in the form of Cellulon fiber was obtained from Weyerhaeuser (Lot# HWO-G005-1, 16% solids, 1 lb on a dry wet basis, DWB). Another sample of Cellulon fiber (~150 g wetcake) was obtained from the NutraSweet Kelco Company. In addition, we considered ordering one or two *A. xylinum* strains from ATCC, and producing bacterial cellulose in the lab. The two most probable candidates will be the strain developed at Cetus Corp. (ATCC #53264) and the strain recently isolated in Japan from black cherry (ATCC #700178), both producing large amounts of cellulose in stirred cultures.

Cotton fiber from American Upland Cotton (*Gossypium hirsutum*) is being obtained from Prof. Thea Wilkins, Agronomy and Range Science, UC Davis. We also have ordered purified dewaxed cotton cellulose 1 AY from Proctor and Gamble Cellulose. As soon as we receive the cotton samples, we will extend the standard procedure to this substrate.

Potential lignocellulosic substrates available in our laboratory include rice straw and mixed urban waste pretreated at NREL with dilute sulfuric acid. Composition of these materials is shown in Table 1. It should be noted, however, that these materials were prepared some time ago, and that freshly prepared lignocellulosic feedstock(s) of interest to the NREL Ethanol Project will be required to perform our tasks on this subcontract.

Table 1.

Pretreated lignocellulosic materials

Material	Glucan (%)	Non-glucose polysaccharides (%)	Amount (gallons)	Solids (%)	Received
Rice straw*	45.1	1.5	3	30	08/95
Mixed urban waste**	54.5	1.0	10	24	06/97

* Pretreated at NREL in a high-solids, pilot scale (100-L) reactor. Conditions: Solids 10%, sulfuric acid 0.8 wt%, 160°C, 10 min

** Prepared at NREL by blending five different feedstocks, fir (35% of dry weight), almond tree pruning (20%), wheat straw (20%), office waste paper (12.5%), and newsprint (12.5%), followed by hydrolysis with dilute sulfuric acid

The standard procedure for measuring cellulase activity as applied to bacterial cellulose is shown in Diagrams 1 – 6. Diagrams 1 – 4 show modifications of the procedure that allows for a separate determination of soluble and insoluble RS in the same hydrolysis mixture. The modifications were necessary in order to accommodate a variety of dilutions of the samples prior to analysis (dilution factor n = 1, 5, 10 or 20). Simplified procedures for measuring only soluble RS or only total (soluble + insoluble) RS are outlined in Diagrams 5 and 6, respectively.

Diagram 7 shows the procedure that was developed in our laboratory earlier and employed low-molecular weight insoluble cellulose, such as Avicel, phosphoric-acid swollen cellulose (PSC) or $ZnCl_2$-treated cellulose (ZTC). The procedure was based on the ability of these materials to form homogeneous suspensions, thus allowing for uniform sampling of the reaction mixtures. As we expected, this procedure could not be directly applied to such materials as cotton fiber, bacterial cellulose or lignocellulosic feedstocks, as the non-homogeneity of their suspensions resulted in large variations in sampling. Instead, we had to develop a completely new procedure that does not rely on our ability to prepare the homogeneous cellulose suspensions or take uniform samples.

Another advantage of the new procedure is that the samples can be diluted to different extents prior to addition of the BCA Reagent, thus broadening the range of the measurable RS concentrations. The samples can also be analyzed for RS without preliminary dilution, thus increasing the sensitivity of the RS determination compared to the previous procedure that required 10-fold dilution of the samples.

Moreover, the new procedure employs glass centrifuge tubes instead of plastic microcentrifuge tubes for enzymatic hydrolysis, thus avoiding a potentially serious problem with cellulase adsorption onto the plastic material.

It should be noted that development of the methods for preparation of the uniform cellulose suspensions is worth pursuing, since it would allow the use of a single reaction mixture for time-course kinetics studies and save time and effort compared to methods that require the preparation of a separate hydrolysis mixture for each time-point of the reaction. However, the methods used to obtain the uniform cellulose suspensions can potentially alter the cellulose properties, may be not equally applicable to all potential cellulosic substrates, and can be energy-consuming.

For example, according to Okiyama *et al* (1993), preparation of a bacterial cellulose suspension should include a crushing process and a homogenizing operation. It was shown, that in order to maintain the hydrophilic property of bacterial cellulose, the crushing process must be carried out in aqueous phase and as far as possible without cutting the microfibrils. For this purpose, cutting with a sharp edge was not suitable, and beating with a dull edge was recommended. As for the homogenizing procedure, the selection of homogenizing apparatus was important. No significant effects were observed following treatment with a commercial disperser, colloid-mill, ball-mill or ultrasonic pulverizer, and only the high pressure homogenizing operation, which required a lot of energy, was effective.

Pertaining to **Task 2** of this subcontract, the BCA method has to be adapted to characterizing endoglucanases selected by the NREL Research Monitor on insoluble high-molecular weight cellulose and lignocellulose.

Our recent inventory of purified cellulases, which include *Trichoderma reesei* EGI, EGII and CBHI; *Acidothermus cellulolyticus* E1, *Thermomonospora fusca* E1, E2, E4 and E5; and *Cellulomonas fimi* CenA, CenB, CenC, CenD, Cex and CbhA is given in Table 2. It should be noted, that most of the purified enzymes that we have, were obtained between 1992-1995. We have only limited quantities of some enzymes and we have noted that the activity of some of the enzyme preparations have significantly decreased over time. We assume that in order to accomplish Task 2, we will need new purified cellulases selected and sent to UC Davis by the NREL Research Monitor. We suggest beginning with *Trichoderma reesei* enzymes (EGI, EGII, CBHI and CBHII) and later increase the number, as engineered cellulases are available for evaluation.

Pertaining to **Task 4**, the HPSEC-MALLS method has to be developed for analysis of insoluble high-molecular weight cellulose using DMAc/LiCl solvent system.

We are conducting an extensive search of the literature for methods and techniques used to dissolve cellulose in DMAc/LiCl. The results of this work will be summarized and presented to NREL in the subsequent technical status reports. At this point, the dissolution method described by Timpa (1991) appears to be the most promising of the literature procedures since it was successful for preparation of high-molecular weight cotton fiber samples for GPC analysis. It is also interesting to note that Einfeldt and

Klemm (1997) were able to obtain highly viscous 0.5% solutions of bacterial cellulose from *Acetobacter xylinum* by direct (without previous activation) dissolving of freeze-dried samples with ~3% water content in DMAc/LiCl at room temperature. We will test both methods.

We are also preparing a table summarizing conditions used by different research groups for GPC analysis of cellulose in DMAc/LiCl solvent system. The table will be presented in the subsequent technical status reports.

Pertaining to **Task 5**, the HPSEC-MALLS method has to be applied to follow the changes in the MWD of insoluble cellulose substrates during hydrolysis with selected endoglucanases and exoglucanases.

It should be noted that, to our knowledge, the HPSEC-MALLS analysis of cellulose in DMAc/LiCl solvent system has never been applied to study the kinetics of enzymatic hydrolysis of insoluble cellulose. According to the search of literature that we conducted and summarized in Table 3, all three articles describing the changes in the MWD of insoluble cellulose under action of purified enzymes employed HPSEC of tricarbanilate derivatives in tetrahydrofuran (THF) for analysis of cellulose (Kleman-Leyer *et al.*, 1994, Kleman-Leyer *et al*., 1996, Srisodsuk *et al*., 1998).

References

Einfeldt, L. and Klemm, D. The control of cellulose biosynthesis by *Acetobacter xylinum* in view of molecular weight distribution. Part I: Change of molecular weight of bacterial cellulose by simple variation of culture conditions. *J. Carbohydr. Chem.* 1997, **16**(4&5), 635-646.

Kleman-Leyer, K. M., Gilkes, N. R., Miller Jr., R. C. and Kent Kirk, T. Changes in the molecular-size distribution of insoluble celluloses by the action of recombinant *Cellulomonas fimi* cellulases. *Biochem. J.* 1994, **302**, 463-469.

Kleman-Leyer, K. M., Siika-Aho, M., Teeri, T.T. and Kent Kirk, T. The cellulases endoglucanase I and cellobiohydrolase II of *Trichoderma reesei* act synergistically to solubilize native cotton cellulose but not to decrease its molecular size. *Appl. Environ. Microbiol.* 1996, **62(8)**, 2883-2887.

Okiyama, A., Motoki, M. and Yamanaka, S. Bacterial cellulose. 3. Development of a new form of cellulose. *Food Hydrocolloids.* 1993, **6**(6), 493-501.

Srisodsuk, M., Kleman-Leyer, K., Keranen, S., Kent Kirk, T. and Teeri, T.T. Modes of action on cotton and bacterial cellulose of a homologous endoglucanase-exoglucanase pair from *Trichoderma reesei. Eur. J. Biochem.* 1998, **251**, 885-892.

Timpa, J. D. Application of universal calibration in gel permeation chromatography for molecular weight determinations of plant cell wall polymers: Cotton fiber. *J. Agric. Food Chem.* 1991, **39**, 270-275.

Table 2
Purified cellulases

Enzyme	Source	Protein	Buffer	Obtained from	Date	Amount	Storage
EGI	*Trichoderma reesei*	5.6 mg/ml		Genencor	7/9/92	~0.2 ml	4°C
EGI	*T. reesei*	11.2 mg/ml	0.05 M Na-acetate, pH 5.0	H. Ding, UCD	12/28/98	~5.5 ml	-20°C
EGII	*T. reesei*	6 mg/ml		Genencor	7/9/92	~0.3 ml	4°C
CBHI	*T. reesei*	7.2 mg/ml	0.05 M Na-acetate, pH 5.0	UCD	12/3/98	~8.5 ml	-20°C
E1	*Acidothermus cellulolyticus*	20 μg/ml		M. Himmel, NREL	5/18/93, 2nd lot	~0.4 ml	4°C
E1	*A. cellulolyticus*	100 μg/ml	0.05 M Na-acetate, pH 5.0	NREL	3/13/95	~0.1 ml	4°C
E1	*A. cellulolyticus*	142.9 μg/ml	20 mM Bis-Tris, pH 5.8	NREL	4/16/93	~0.05 ml	4°C
E1	*A. cellulolyticus*	178 μg/ml	20 mM Bis-Tris, 100 mM NaCl, pH 5.8	NREL	5/17/93	~0.05 ml	4°C
E1	*Thermomonospora fusca*	100 μg/ml	0.05 M Na-acetate, pH 5.0	D. Wilson, Cornell	3/13/95	~2.0 ml	-20°C
E2	*T. fusca*	100 μg/ml	0.05 M Na-acetate, pH 5.0	Cornell	3/13/95	~0.1 ml	-20°C
E4	*T. fusca*	100 μg/ml	0.05 M Na-acetate, pH 5.0	Cornell	3/13/95	~2.5 ml	-20°C
E5	*T. fusca*	100 μg/ml	0.05 M Na-acetate, pH 5.0	Cornell	3/13/95	~0.5 ml	-20°C
E5	*T. fusca*	100 μg/ml	0.05 M Na-acetate, pH 5.0	Cornell	3/13/95	~1.0 ml	-20°C
CenA	*Cellulomonas fimi*	1 mg/ml		D. Kilburn, UBC	1993	~0.5 ml	4°C
CenB	*C. fimi*	1 mg/ml		UBC	1993	~0.3 ml	4°C
CenC	*C. fimi*	0.73 mg/ml ~5.63 μM ?	50 mM Na-phosphate, 0.02% NaN_3, pH 7.0	UBC	1993	~0.2 ml	4°C
CenD	*C. fimi*	2 mg/ml	50 mM Na-phosphate, 0.02% NaN_3, pH 7.0	UBC	1993	~0.5 ml	4°C
Cex	*C. fimi*	2 mg/ml		UBC	1993	~0.4 ml	4°C
CbhA	*C. fimi*	5 mg/ml		UBC	1993	~0.4 ml	4°C

Table 3.
Changes in MWD of insoluble cellulose during enzymatic hydrolysis

Reference	Substrates	Enzymes	Hydrolysis	Analytical Methods	Analyzed Parameters	Conclusions
Chang, M. M., Chang, T. Y.C. and Tsao, G. T. *Adv. Biochem. Eng.* 1981, **20**, 15-42	Mechanically modified cotton fibers	Culture filtrate of *Trichoderma reesei*				Dramatic shifts in the MWD of cellulose were observed
Puls, J. In: *Energy from Biomass 1* (Grassi, G. and Zibetta, H., eds.). Elsevier Applied Science, London, NY, 1987, 224-229	Steam-pretreated birchwood	Culture filtrate of *T. reesei*				Reduction of the average chain length of carbohydrates was observed
Puls, J. and Wood, T. M. *Bioresource Technol.* 1991, **36**, 15-19	Birchwood cellulose	Culture filtrates *Neocallimastix frontalis, Trichoderma koningii, Penicillium pinophilum, Clostridium thermocellum*				All culture filtrates except for *C. thermocellum* reduced the chain length of birchwood cellulose to various extents.
Kleman-Leyer, K., Agosin, E., Conner, A. H., and Kent Kirk, T. Changes in molecular size distribution of cellulose during attack by white rot and brown rot fungi. *Appl. Environ. Microbiology*. 1992, **58(4)**, 1266-1270	Cotton cellulose, Type A-600 (Holden Vale Manufacturing Co., Ltd., Lancaster, England)	Brown rot fungus *Postia placenta* White rot fungus *Phanerochaete chrysosporium*	Cellulose hydrolysis in solid-state cultures (Ref)	Viscometry of cellulose solution in cupriethylenediamine (ASTM Standards, 1981) SEC of tricarbanilate derivatives dissolved in tetrahydrofuran (THF) (Wood *et al.*, 1986) Weighing of residual cellulose	DP MWD, DP_n, DP_w, DP_z Weight loss	The brown rot fungus cleaves completely through the amorphous regions of the cellulose microfibrils, whereas the white rot fungus attacked the surfaces of the microfibrils, resulting in a progressive erosion
Kleman-Leyer, K. M., Gilkes, N. R., Miller Jr., R. C. and Kent Kirk, T. Changes in the molecular-size distribution of insoluble celluloses by the action of recombinant *Cellulomonas fimi* cellulases. *Biochem. J*. 1994, **302**, 463-469	Purified dewaxed cotton cellulose 1AY (Procter and Gamble Cellulose, Memphis, TN, USA) Bacterial cellulose from *Acetobacter xylinum* ATCC 53524 (Hackney *et al.*, 1993) Bacterial microcrystalline cellulose (BMCC) from *A. xylinum* ATCC 23769 (Gilkes *et al.*, 1992)	Recombinant cellulases of *Cellulomonas fimi* (ATCC 484)*:* Endoglucanase CenA Isolated CD of CenA CenB CenD Cex	Cellulose hydrolysis @ 39°C, stirring	HPSEC of tricarbanilate derivatives in tetrahydrofuran (THF) (Wood *et al.*, 1986) Phenol/H_2SO_4 assay (Chaplin & Kennedy, 1986)	MWD DP_n, DP_w Total sugars in supernatants and weight loss	CenA attacks cellulose by preferentially cleaving completely through the cellulose microfibrils at the amorphous regions, and much more slowly by degrading the crystalline surfaces. CenB and CenD cleave the amorphous regions much less efficiently while vigorously degrading the surfaces of the crystalline regions of the microfibrils

Reference	Substrates	Enzymes	Hydrolysis	Analytical Methods	Analyzed Parameters	Conclusions
Kleman-Leyer, K. M., Siika-Aho, M., Teeri, T.T. and Kent Kirk, T. The cellulases endoglucanase I and cellobiohydrolase II of *Trichoderma reesei* act synergistically to solubilize native cotton cellulose but not to decrease its molecular size *Appl. Environ. Microbiol.* 1996, **62(8)**, 2883-2887	Purified dewaxed cotton cellulose 1AY (Procter and Gamble Cellulose, Memphis, TN, USA) Bacterial cellulose was produced by *A. xylinum* ATCC 12733 grown on PYD medium (Hestrin, 1963). BMCC was prepared as described by Gilkes *et al*, 1992	EGI from T. *reesei* strain that does not produce CBHI or CBHII (Oy Alko Ab, Helsinki, Finland) CBHII from *T. reesei* RUT-C30	Hydrolysis of cellulose (10 mg/ml) with 2 nmol of EGI alone or in combination with 2 nmol of CBHII @ 39°C, pH 5.0, stirring Samples were centrifuged @ 16,000 g for 6-8 min. The pellet was washed with water (3x1.5 ml), oven-dried at 60°C and used for MWD determination by SEC	HPSEC of tricarbanilate derivatives in tetrahydrofuran (THF) (Wood *et al.*, 1986). Calibration with narrow polysterene standards using the universal calibration technique (Yau *et al.*, 1980) DNS method (Wood & Saddler, 1988) Phenol/H_2SO_4 assay (Chaplin & Kennedy, 1986)	MWD DP_n, DP_w Reducing sugars Total sugars and weight loss	EGI degrades cotton cellulose by selectively cleaving through the microfibrils at the amorphous sites, whereas CBHII releases soluble sugars from the EGI-degraded cotton cellulose and from the more crystalline BMCC
Srisodsuk, M., Kleman-Leyer, K., Keranen, S., Kent Kirk, T. and Teeri, T.T. Modes of action on cotton and bacterial cellulose of a homologous endoglucanase-exoglucanase pair from *Trichoderma reesei* *Eur. J. Biochem*. 1998, **251**, 885-892	Purified dewaxed cotton cellulose 1AY (Procter and Gamble Cellulose, Memphis, TN, USA) BMCC from *A. xylinum* was produced and prepared as described by Hestrin, 1963; Gilkes *et al*, 1992	EGI and CBHI from *T. reesei* and their corresponding catalytic domains	As described in Kleman-Layer *et al*, 1996	HPSEC of tricarbanilate derivatives in tetrahydrofuran (THF) (Wood *et al.*, 1986). Calibration with narrow polysterene standards using the universal calibration technique (Yau *et al.*, 1980 DNS method (Wood & Saddler, 1988) Phenol/H_2SO_4 assay (Chaplin & Kennedy, 1986)	MWD DP_n, DP_w Reducing sugars Total sugars and weight loss	The action of CBHI on BMCC results in efficient solubilization but only a slow decrease in DP. In contrast, the action of EGI in a rapid decrease in the DP but less efficient overall solubilization of the substrate. CBHI alone was practically inactive toward cotton, while EGI rapidly reduced the DP of cotton, and slowly solubilised part of it. Working synergistically, EGI and CBHI solubilised cotton more rapidly and to a greater extent than EGI alone

Standard procedure for measurement of cellulase activity on insoluble high-molecular weight cellulose using the BCA method for reducing sugar determination

This procedure was developed to measure the activity of cellulases on insoluble high-molecular weight cellulose. The procedure employs the disodium 2,2'-bicinchoninate (BCA) method for highly sensitive determination of reducing sugars (RS) in the hydrolysis mixtures. The procedure allows for reliable measurement of the initial rate of hydrolysis corresponding to 0.01 - 5% of substrate conversion. Both soluble and insoluble RS can be measured using the same hydrolysis mixture (Diagrams 1 - 4), which allows ranking cellulases according to endo- or exo-mode of action. Simplified procedure for measuring only soluble or only total (soluble + insoluble) RS is shown on Diagrams 5 and 6.

Reagents

Reagent A is prepared by dissolving 54.28 g/L (512 mM) Na_2CO_3, 24.2 g/L (288 mM) $NaHCO_3$ and 1.942 g/L (5 mM) disodium 2,2'-bicinchoninate in DI water. The solution is stored in an amber bottle at room temperature for several months.

Reagent B is prepared by dissolving 1.248 g/L (5 mM) $CuSO_4x5\ H_2O$ and 1.262 g/L (12 mM) L-serine in DI water. The solution is stored in an amber bottle at 4ºC for up to 2 months.

BCA Reagent is prepared by mixing equal volumes of Reagents A and B on the day of analysis.

Alkaline Solution (pH 9.9) is prepared by dissolving 54.28 g/L (512 mM) Na_2CO_3, and 24.2 g/L (288 mM) $NaHCO_3$ in DI water.

Standard glucose solutions in the range of 0.5 - 10.0 µg/ml (2.78 - 55.6 nmol/ml) are prepared from the stock glucose solution (50 µg/ml) by dilution with DI water.

Enzymatic hydrolysis

Enzymatic hydrolysis of bacterial cellulose from *Acetobacter xylinum* is carried out in 0.05 M Na-acetate buffer, pH 5.0, at 40°C under constant stirring (100 rpm). The Initial Reaction Mixture contains 4 mg/ml cellulose (on a dry weight basis) and 10 to 500 ng/ml of EGI from *Trichoderma reesei* (see Diagrams 1-4).

A wet cellulose sample containing the necessary amount of dry cellulose is placed in a 10-ml glass centrifuge tube (Pyrex) and, after addition of the buffer, equilibrated in a water bath shaker at 40°C and 100 rpm for 10 min. The reaction is started by addition of 20 to 100 µl of enzyme solution, which is prepared immediately before the hydrolysis by diluting stock enzyme solution with 0.05 M Na-acetate buffer (pH 5.0). The stock

enzyme solution is stored at 4°C. A control reaction mixture is prepared in a similar way, but contains no enzyme.

The total volume of the Initial Reaction Mixture is 1.0 ml or 2.0 ml depending on the protocol used (see Diagrams 1-4 for details). The volume of the liquid phase in the wet cellulose sample should taken into account when calculating the amount of buffer and enzyme to be added to the reaction mixture.

The centrifuge tube is sealed with Parafilm and incubated in a water bath shaker at 40°C and 100 rpm. At the designated time-point, the reaction is stopped by adding the Alkaline Solution (pH 9.9), which inactivates the enzyme.

Inactivated Reaction Mixture, containing 4 vol. of the Initial Reaction Mixture and 1 vol. of the Alkaline Solution, is either immediately analyzed for RS using the BCA method as described below, or stored at 4°C for subsequent analysis.

BCA analysis

Inactivated Reaction Mixture is centrifuged for 5 min at 4,800 rpm using a Beckman GS-15R centrifuge. A specified volume of the supernatant (Liquid Fraction) is transferred into another test tube (see Diagrams 1-4 of the BCA Procedure for details), while the precipitate in the first test tube is thoroughly mixed with the remaining solution to form the Suspension. The volume of the Suspension is calculated as the difference between the volumes of the Inactivated Reaction Mixture and the Liquid Fraction ($V_{susp} = V_{inact} - V_{liq}$). Reducing sugars are determined in the Liquid Fraction and the Suspension by the BCA method following the appropriate dilution of both samples with DI water as shown in Diagrams 1 - 4.

The BCA Reagent is added to the diluted samples, so that the resulting mixture contains 5 vol. of the diluted sample and 4 vol. of the BCA Reagent (see Diagrams 1-4). The tubes are stirred, covered with marbles to minimize evaporation, and incubated at 80°C for 45 min. Following the incubation, the tubes are cooled in a water bath to room temperature. The tube containing the diluted sample of Suspension is thoroughly stirred and then centrifuged for 5 min at 4,800 rpm to obtain a particle-free supernatant.

Both solutions are sealed with Parafilm and mixed by inverting several times. The absorbance at 560 nm (A_{560}) is then measured against a blank using a Model UV160U spectrophotometer (Shimadzu, Japan). RS concentrations in diluted samples ($[RS]_{dil.liq.}$ and $[RS]_{dil.susp.}$) are determined from the calibration curve, which is obtained using standard glucose solutions in the range of 0.5 - 10.0 μg/ml (2.78 - 55.6 nmol/ml). To obtain the calibration curve, standard glucose solutions are mixed with the BCA reagent as shown in Table 4 (Glc + BCA Reagent → Incubate at 80°C for 45 min → Cool to room temp. → Measure A_{560}). The blank is prepared as shown in Table 4 and contains DI water instead of analyzed solutions (DI water + BCA Reagent → Incubate at 80°C for 45 min → Cool to room temp.).

Concentration of soluble, insoluble, and total (soluble + insoluble) RS in the Initial Reaction Mixture in nmol/ml is calculated using the following equations:

$$[RS]_{soluble} = [RS]_{dil.liq.} * n * V_{inact} / V_o$$

$$[RS]_{insoluble} = ([RS]_{dil.susp.} - [RS]_{dil.liq.}) * n * V_{susp} / V_0$$

$$[RS]_{total} = [RS]_{soluble} + [RS]_{insoluble} = n * ([RS]_{dil.liq.} * V_{liq.} + [RS]_{dil.susp.} * V_{susp}) / V_o$$

Determination of cellulase activity

After kinetic curves "RS vs. reaction time" are obtained for several concentrations of the enzyme, the initial rate of hydrolysis is determined for each enzyme concentration as the slope of the regression line plotted in the range of linearity, $V_o^{RS} = dRS/dt_{\ t \to 0}$, $nmol_{RS}/(ml*min)$.

The initial rates of hydrolysis are then plotted as a function of the enzyme concentration, and the specific activity is calculated as the slope of the regression line in the range of linearity, $A_{sp} = dV_o^{RS}/dE_{\ E \to 0}$. Specific activity should be expressed as $nmol_{RS}/(min*ng_E)$ or as international units IU (μmol of glucosidic bonds hydrolyzed in one min during the initial period of hydrolysis) per mg protein:

$$IU/mg_E = \mu mol_{RS}/(min*mg_E) = 10^3 * nmol_{RS}/(min*ng_E)$$

To perform all measurements in duplicate, two reaction mixtures should be prepared and subsequently analyzed for each time point of hydrolysis. Reducing sugars in the above equations can be $RS_{soluble}$, $RS_{insoluble}$, or RS_{total}.

Diagram 1

Outline of the Procedure for Measurement of Cellulase Activity on Insoluble High-Molecular Weight Cellulose Using BCA Method for RS Determination (No dilution, n = 1)

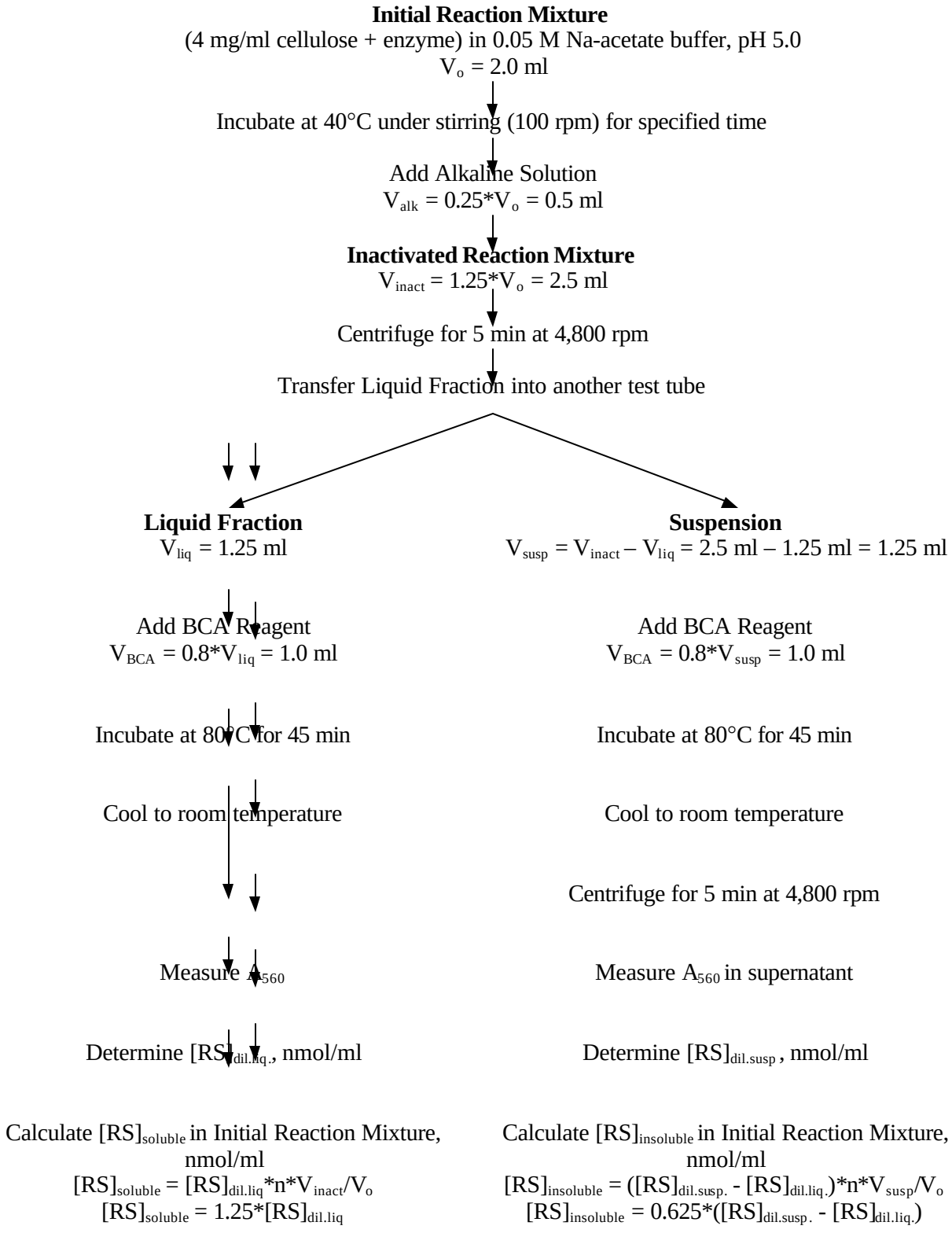

Diagram 2

Outline of the Procedure for Measurement of Cellulase Activity on Insoluble High-Molecular Weight Cellulose Using BCA Method for RS Determination (Dilution n = 5)

Initial Reaction Mixture
(4 mg/ml cellulose + enzyme) in 0.05 M Na-acetate buffer, pH 5.0
V_o = 2.0 ml
↓
Incubate at 40°C under stirring (100 rpm) for specified time
↓
Add Alkaline Solution
$V_{alk} = 0.25*V_o$ = 0.5 ml
↓
Inactivated Reaction Mixture
$V_{inact} = 1.25*V_o$ = 2.5 ml
↓
Centrifuge for 5 min at 4,800 rpm
↓
Transfer Liquid Fraction into another test tube

Liquid Fraction	**Suspension**
V_{liq} = 2.0 ml	$V_{susp} = V_{inact} - V_{liq}$ = 2.5 ml – 2.0 ml = 0.5 ml
↓	
Transfer part of Liquid Fraction into another test tube	
$V_{trans.liq.} = V_{susp}$ = 0.5 ml	
↓	
Dilute sample 5-fold by adding 2.0 ml water (n = 5)	Dilute suspension 5-fold by adding 2.0 ml water (n = 5)
$V_{dil.liq.}$ = 2.5 ml	$V_{dil.susp.}$ = 2.5 ml
↓	↓
Add BCA Reagent	Add BCA Reagent
$V_{BCA} = 0.8*V_{dil.liq.}$ = 2.0 ml	$V_{BCA} = 0.8*V_{dil.susp.}$ = 2.0 ml
↓	↓
Incubate at 80°C for 45 min	Incubate at 80°C for 45 min
↓	↓
Cool to room temperature	Cool to room temperature
	↓
	Centrifuge for 5 min at 4,800 rpm
↓	↓
Measure A_{560}	Measure A_{560} in supernatant
↓	↓
Determine $[RS]_{dil.liq.}$, nmol/ml	Determine $[RS]_{dil.susp.}$, nmol/ml
↓	↓
Calculate $[RS]_{soluble}$ in Initial Reaction Mixture, nmol/ml	Calculate $[RS]_{insoluble}$ in Initial Reaction Mixture, nmol/ml

$$[RS]_{soluble} = [RS]_{dil.liq.} * n * V_{inact} / V_o$$

$$[RS]_{soluble} = 6.25 * [RS]_{dil.liq.}$$

$$[RS]_{insoluble} = ([RS]_{dil.susp.} - [RS]_{dil.liq.}) * n * V_{susp} / V_o$$

$$[RS]_{insoluble} = 1.25 * ([RS]_{dil.susp.} - [RS]_{dil.liq.})$$

Diagram 3

Outline of the Procedure for Measurement of Cellulase Activity on Insoluble High-Molecular Weight Cellulose Using BCA Method for RS Determination (Dilution n = 10)

Initial Reaction Mixture
(4 mg/ml cellulose + enzyme) in 0.05 M Na-acetate buffer, pH 5.0
V_o = 2.0 ml
↓
Incubate at 40°C under stirring (100 rpm) for specified time
↓
Add Alkaline Solution
$V_{alk} = 0.25*V_o$ = 0.5 ml
↓
Inactivated Reaction Mixture
$V_{inact} = 1.25*V_o$ = 2.5 ml
↓
Centrifuge for 5 min at 4,800 rpm
↓
Transfer Liquid Fraction into another test tube

Liquid Fraction	**Suspension**
V_{liq} = 2.0 ml	$V_{susp} = V_{inact} – V_{liq}$ = 2.5 ml – 2.0 ml = 0.5 ml
↓	
Transfer part of Liquid Fraction into another test tube	
$V_{trans.liq.} = V_{susp}$ = 0.5 ml	
↓	
Dilute sample 10-fold by adding 4.5 ml water (n = 10)	Dilute suspension 10-fold by adding 4.5 ml water (n = 10)
$V_{dil.liq.}$ = 5.0 ml	$V_{dil.susp.}$ = 5.0 ml
↓	↓
Add BCA Reagent	Add BCA Reagent
$V_{BCA} = 0.8*V_{dil.liq.}$ = 4.0 ml	$V_{BCA} = 0.8*V_{dil.susp.}$ = 4.0 ml
↓	↓
Incubate at 80°C for 45 min	Incubate at 80°C for 45 min
↓	↓
Cool to room temperature	Cool to room temperature
	↓
	Centrifuge for 5 min at 4,800 rpm
↓	↓
Measure A_{560}	Measure A_{560} in supernatant
↓	↓
Determine $[RS]_{dil.liq.}$, nmol/ml	Determine $[RS]_{dil.susp.}$, nmol/ml
↓	↓
Calculate $[RS]_{soluble}$ in Initial Reaction Mixture, nmol/ml	Calculate $[RS]_{insoluble}$ in Initial Reaction Mixture, nmol/ml
$[RS]_{soluble} = [RS]_{dil.liq.}*n*V_{inact}/V_o$	$[RS]_{insoluble} = ([RS]_{dil.susp.} - [RS]_{dil.liq.})*n*V_{susp}/V_o$

$[RS]_{soluble} = 12.5*[RS]_{dil.liq.}$

$[RS]_{insoluble} = 2.5*([RS]_{dil.susp.} - [RS]_{dil.liq.})$

Diagram 4

Outline of the Procedure for Measurement of Cellulase Activity on Insoluble High-Molecular Weight Cellulose Using BCA Method for RS Determination (Dilution n = 20)

Initial Reaction Mixture
(4 mg/ml cellulose + enzyme) in 0.05 M Na-acetate buffer, pH 5.0
V_o = 1.0 ml
↓
Incubate at 40°C under stirring (100 rpm) for specified time
↓
Add Alkaline Solution
$V_{alk} = 0.25*V_o$ = 0.25 ml
↓
Inactivated Reaction Mixture
$V_{inact} = 1.25*V_o$ = 1.25 ml
↓
Centrifuge for 5 min at 4,800 rpm
↓
Transfer Liquid Fraction into another test tube

Liquid Fraction	**Suspension**
V_{liq} = 1.0 ml	$V_{susp} = V_{inact} – V_{liq}$ = 1.25 ml – 1.0 ml = 0.25 ml
↓	
Transfer part of Liquid Fraction into another test tube	
$V_{trans.liq.} = V_{susp}$ = 0.25 ml	
↓	
Dilute sample 20-fold by adding 4.75 ml water (n = 20)	Dilute suspension 20-fold by adding 4.75 ml water (n = 20)
$V_{dil.liq.}$ = 5.0 ml	$V_{dil.susp.}$ = 5.0 ml
↓	↓
Add BCA Reagent	Add BCA Reagent
$V_{BCA} = 0.8*V_{dil.liq.}$ = 4.0 ml	$V_{BCA} = 0.8*V_{dil.susp.}$ = 4.0 ml
↓	↓
Incubate at 80°C for 45 min	Incubate at 80°C for 45 min
↓	↓
Cool to room temperature	Cool to room temperature
	↓
	Centrifuge for 5 min at 4,800 rpm
↓	↓
Measure A_{560}	Measure A_{560} in supernatant
↓	↓
Determine $[RS]_{dil.liq.}$, nmol/ml	Determine $[RS]_{dil.susp.}$, nmol/ml
↓	↓
Calculate $[RS]_{soluble}$ in Initial Reaction Mixture, nmol/ml	Calculate $[RS]_{insoluble}$ in Initial Reaction Mixture, nmol/ml
$[RS]_{soluble} = [RS]_{dil.liq.}*n*V_{inact}/V_o$	$[RS]_{insoluble} = ([RS]_{dil.susp.} - [RS]_{dil.liq.})*n*V_{susp}/V_o$

$[RS]_{soluble} = 25*[RS]_{dil.liq.}$ $[RS]_{insoluble} = 5*([RS]_{dil.susp.} - [RS]_{dil.liq.})$

Table 4

Preparation of the Standard Glucose Solutions and Blank Solutions for the protocols shown in Diagrams 1 – 4

	No dilution before adding the BCA Reagent (Diagram 1)	**5-fold dilution (Diagram 2)**	**10- or 20-fold dilution (Diagrams 3 or 4)**
Standard Glucose Solutions	1.25 ml Glc (2.78 – 55.6 nmol/ml) + 1.0 ml BCA Reagent ↓ Incubate at 80°C for 45 min ↓ Cool to room temp. ↓ Measure A_{560}	2.5 ml Glc (2.78 – 55.6 nmol/ml) + 2.0 ml BCA Reagent ↓ Incubate at 80°C for 45 min ↓ Cool to room temp. ↓ Measure A_{560}	5.0 ml Glc (2.78 – 55.6 nmol/ml) + 4.0 ml BCA Reagent ↓ Incubate at 80°C for 45 min ↓ Cool to room temp. ↓ Measure A_{560}
Blank Solutions	1.25 ml DI water + 1.0 ml BCA Reagent ↓ Incubate at 80°C for 45 min ↓ Cool to room temp.	2.5 ml DI water + 2.0 ml BCA Reagent ↓ Incubate at 80°C for 45 min ↓ Cool to room temp.	5.0 ml DI water + 4.0 ml BCA Reagent ↓ Incubate at 80°C for 45 min ↓ Cool to room temp..

Diagram 5

Outline of the Procedure for Measurement of Cellulase Activity on Insoluble High-Molecular Weight Cellulose Using BCA Method for Determination of Soluble RS (Dilution n)

Initial Reaction Mixture
(4 mg/ml cellulose + enzyme) in 0.05 M Na-acetate buffer, pH 5.0
V_o = 2.0 ml
↓
Incubate at 40°C under stirring (100 rpm) for specified time
↓
Add Alkaline Solution
$V_{alk} = 0.25*V_o$ = 0.5 ml
↓
Inactivated Reaction Mixture
$V_{inact} = 1.25*V_o$ = 2.5 ml
↓
Centrifuge for 5 min at 4,800 rpm
↓
Dilute an aliquot of the Liquid Fraction (25µl – 1.25 ml)
n-fold by adding (n-1)*$V_{aliquot}$ ml water
to total volume of 1.25 ml
$V_{dil.liq.}$ = 1.25 ml
↓
Add BCA Reagent
$V_{BCA} = 0.8*V_{dil.liq.}$ = 1.0 ml
↓
Incubate at 80°C for 45 min
↓
Cool to room temperature
↓
Measure A_{560}
↓
Determine $[RS]_{dil.liq.}$, nmol/ml
↓
Calculate $[RS]_{soluble}$ in Initial Reaction Mixture, nmol/ml
$[RS]_{soluble} = [RS]_{dil.liq.}*n*V_{inact}/V_o$

Diagram 6

Outline of the Procedure for Measurement of Cellulase Activity on Insoluble High-Molecular Weight Cellulose Using BCA Method for Determination of Total RS (Dilution n = 1, 2, or4)

Initial Reaction Mixture
(4 mg/ml cellulose + enzyme) in 0.05 M Na-acetate buffer, pH 5.0
V_o = 1.0 ml
↓
Incubate at 40°C under stirring (100 rpm) for specified time
↓
Add Alkaline Solution
$V_{alk} = 0.25*V_o$ = 0.25 ml
↓
Inactivated Reaction Mixture
$V_{inact} = 1.25*V_o$ = 1.25 ml
↓
Dilute suspension n-fold by adding (n-1)*V_{inact} ml water
(n = 1, 2, or 4)
$V_{dil.susp.} = n*V_{inact}$ = 1.25, 2.5, or 5.0 ml
↓
Add BCA Reagent
$V_{BCA} = 0.8*V_{dil.susp.}$ = 1.0, 2.0, or 4.0 ml
↓
Incubate at 80°C for 45 min
↓
Cool to room temperature
↓
Centrifuge for 5 min at 4,800 rpm
↓
Measure A_{560} in supernatant
↓
Determine $[RS]_{dil.susp.}$, nmol/ml
↓
Calculate $[RS]_{total}$ in Initial Reaction Mixture, nmol/ml
$[RS]_{insoluble} = [RS]_{dil.susp}*n*V_{inact}/V_o$

Diagram 7

Outline of the Procedure for Measurement of Cellulase Activity on Insoluble Low-Molecular Weight Cellulose Using BCA Method for RS Determination (Dilution n = 10)